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(54) Title: COMPOSITION AND METHOD FOR DESTRUCTION OF INFECTIOUS PRION PROTEINS

(57) Abstract: A method and composition for destruction of infectious prion proteins associated with transmissible spongiform encephalopathy (TSE) and/or other prion protein-mediated diseases, by thermal/enzymatic treatment of the infectious prion proteins with a prion-destructive protease. The method and composition are applicable to treatment of tissue containing or contaminated with infectious prion protein strains, or disinfection or sterilization of prion-contaminated articles, such as surgical instruments, kitchen utensils, laboratory tools, etc.

COMPOSITION AND METHOD FOR DESTRUCTION OF INFECTIOUS PRION PROTEINS

DESCRIPTION

Field of the Invention

The present invention generally relates to a composition and method for destruction of infectious prion proteins associated with transmissible spongiform encephalopathy (TSE), e.g., bovine spongiform encephalopathy (BSE) and sheep scrapie. More specifically, the invention relates to application of proteases for destructing infectious prion proteins contained in animal tissues, and/or for disinfecting and sterilizing medical devices and like articles contaminated by such infectious prior proteins.

Background of the Invention

Prion proteins are conformationally anomalous proteins that are associated with infectious neurodegenerative diseases in human as well as non-human mammalian species.

Prion diseases in non-human mammalian species include scrapie (sheep), transmissible mink encepohalopathy (minks), chronic wasting disease (elk, deer), bovine spongiform encephalopathy (BSE) (cows), feline spongiform encephalopathy (cats), and simian spongiform encephalopathy (monkeys).

In humans a variety of neurodegenerative conditions are etiologically associated with prion proteins, including Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker syndrome, fatal insomnia, kuru, and variant Creutzfeldt-Jakob disease. Pathogenesis of human prion diseases is associated with carnivorism (BSE-infected beef, causing new variant Creutzfeldt-Jakob disease), administration of human growth hormone (causing iatrogenic Creutzfeldt-Jakob disease) and ritualistic cannibalism (causing kuru).

Over 180,000 BSE cases and 100 human Creutzfeldt-Jakob disease cases have been reported in Europe since 1992, and the human cases are predicted to significantly rise. The spread of such disease is difficult to contain, since such disease has no cure and the pathogenic prion protein is recalcitrant and non-immunogenic. The pathogenic and infectious isoform of prion protein is very stable, rich in β-sheet structure, and resistant to heat and common proteolytic enzymes (Prusiner, S.B., *Proc. Natl. Acad. Sci.* U.S.A., 95, 11363 (1998); Cohen, F.E. and Prusiner, S.B., *Ann. Rev. Biochem.*, 67, 793 (1998); and Pan, K-M, Baldwin, M., Nguyen, J., Gasset, M., Serban, A., Groth, D., Mehlhorn, I., Huang, Z., Fletterick, R.J., Cohen, F.E., and Prusiner, S.B., *Proc. Natl. Acad. Sci.* U.S.A, 90, 10962 (1993)).

Significant efforts have been focused on studies of BSE and prion protein contamination of human food supplies deriving from bovine sources, and of prion protein disease generation and propagation in bovine species. Infection in bovine populations has been associated with feeding of bovine herds with feedstocks containing bone meal and rendered organs and tissue deriving from infected cows, sheep and other ruminant animals.

At present, in many countries, animal products that otherwise would provide human food, and animal by-product that otherwise would provide a viable source of raw materials and nutritional supplements for animal feeds, are being incinerated and the ash residue buried, to preclude transmission of prion protein infection deriving from the presence of infectious prion proteins in associated animals.

In Europe, meat bone meal from animal by-products has been banned for feed use. In the United States, no outbreak of BSE has been reported, however, animal and rendering industries have been placed under restrictive regulations to prevent the incidence and spread of disease (Franco, B.A., *Feed Stuffs*, February 12, 2001). Further, the United States has banned imports of meat and meat by-products.

A variety of tests for determining the presence of infectious prion proteins in animal tissue have been developed, including Western blot tests, sandwich immunoassay tests, ELISA tests, fluoroimmunoassay tests, capillary immuno-electrophoresis tests, and plasminogen binding tests (Genetic Engineering News,

Vol. 21, No. 6, March 15, 2001), but corresponding capability for industrially-applicable removal of infectious prion proteins from infected animal tissue has not evolved to date.

Infectious prion proteins are resistant to destruction by conventional methods that denature and otherwise degrade conformationally normal proteins, including methods such as autoclaving (even temperatures as high as 200°C are not effective to inactivate infectious prion proteins), boiling, freezing, and exposure to reagents such as formaldehyde, carbolic acid and chloroform. Typically, incineration or treatment with bleach is employed to destroy the pathogenic isoform of the prion protein.

It therefore would be a significant advance in the art to provide a composition and methodology for destruction of infectious prion proteins, which is applicable to the treatment of biological materials, e.g., animal tissue containing or contaminated with infectious prion proteins.

Moreover, the cross-contamination caused by reuse of medical instruments that have been previously exposed to prion-infected tissues is becoming an increased hazard and potential contributor to the transmission of infection.

The use of antiseptics, disinfectants, and sterilization procedures in health care facilities is critical to prevent the cross-contamination by medical instruments used during health care procedures. Disinfection and sterilization of medical devices or instruments are achieved by a variety of conventional methods, using various physical and chemical processes that destroy infectious biological materials, such as bacteria or viruses. For example, chemical disinfectants such as peracetic acid, hydrogen peroxide, sodium hydroxide, formic acid, bleach, alcohols, ethylene oxide, formaldehyde, formalin, and glutaraldehyde can be used for disinfecting and sterilizing medical devices; incineration, autoclaving, freezing, dry heating, boiling, UV and microwave radiation are also useful for destructing traditional infectious agents such as bacteria and viruses.

However, as discussed hereinabove, infectious prion proteins are known to be resistant to destruction by the conventional methods, which are therefore ineffective for disinfecting or sterilizing prion-contaminated medical devices or similar articles.

It is therefore another object of the present invention to provide a composition and methodology for effectively disinfecting or sterilizing prion-infected medical devices such as surgical instruments, or like articles such as kitchen utensils and laboratory tools.

SUMMARY OF THE INVENTION

The invention provides a method and composition for destruction of infectious prion proteins.

In one aspect, the present invention relates to a method of treatment for reduction of infective prion protein at a locus contaminated with infective prion protein, the method comprising the steps of:

(a) heating the locus to a sufficient temperature and for sufficient time to enhance the proteolytic susceptibility of infective prion protein at the locus; and

(b) exposing the heated locus to a proteolytic enzyme that is effective for at least partial reduction of the infective protein prion at such locus.

Said locus can be either tissue containing or contaminated by infective prion protein therein, or article(s) that are susceptible to contamination by infectious prion protein.

The temperature in step (a) does not exceed about 150°C, and preferably within a range of from about 100°C to about 150°C, more preferably within a range of from about 125°C to about 140°C.

Step (b) is carried out at a temperature that is lower than that of step (a), for example, within a range of: (1) from about 35°C to about 100°C, (2) from about 40°C to abut 75°C, and (3) from about 50°C to about

60°C. Preferably, step (b) is carried out at a temperature above about 40°C, or more preferably, above about 50°C.

The proteolytic enzyme used in step (b) include, but are not limited to, keratinase enzymes, proteinase K, trypsins, chymotrypsins, pepsins, chymosins, cathepsins, subtilisins, elastases, collagenases, endopeptidases, peptidases, oligopeptidease, thermolysins, bacillolysin, mycilysins, carboxypeptidases, leucyl aminopeptidases, aminopeptidases, extremthermophilic proteases, carbonyl hydrolase, papain, pancreatin, streptokinase, streptodornase, ficin, carboxypeptidase, chymopapain, and bromelin. Keratinase enzymes and/or an active fragment thereof are particularly preferred for the practice of the present invention. Keratinases are a group of proteolytic enzymes that are generally known as being capable of breaking down keratin proteins that are the major components of feather, horn, hooves, and hair. The inventor of the present application has discovered an unexpected and surprising result that keratinase enzymes are also effective in destructing infectious prion proteins, especially if the infectious prion proteins have been rendered proteolyticly susceptible. More preferably, such proteolytic enzyme is a Bacillus licheniformis PWD-1 keratinase enzyme and/or an active fragment thereof. Alternatively, such proteolytic enzyme is a mutant of wild-type Bacillus amyloliquefaciens subtilisin, comprising one or more amino acid substitutions, additions, or deletions.

Moreover, such method as described hereinabove may further comprise a step of (c) testing the locus to verify reduction of infective prion protein therein, which comprises subject the locus to a test selected from the group consisting of Western blot tests, sandwich immunoassay tests, ELISA tests, fluoroimmunoassay tests, capillary immuno-electrophoresis tests, and plasminogen binding tests. Western blot test is preferred in practicing the present invention.

One specific aspect of the present invention relates to a method of treatment for reduction of infective prion protein at a locus contaminated or suspected of being contaminated with infective prion protein, as described hereinabove, wherein such locus comprises tissue containing or contaminated by infective prion

protein therein. Such tissue may comprise animal tissue, preferably mammalian tissue such as bovine tissue, ovine tissue, etc., and it can be from any body parts of the animal, such as brain, pituitary, intestine, lung, heart, kidney, and spleen tissues. Preferably, such animal tissue comprises nervous system tissue, and more preferably comprises BSE-infected or scrapie-infected tissue. It can be obtained from a carrier animal for the infective prion protein.

Another specific aspect of the present invention relates to a method of treatment for reduction of infective prion protein at a locus contaminated or suspected of being contaminated with infective prion protein, as described hereinabove, wherein such locus comprises article(s) that a susceptible to contamination by infectious prion protein. Such article(s) may comprise surgical instrument(s), such as clamps, forceps, scissors, knives, cables, punches, tweezers, cannulae, calipers, carvers, curettes, scalers, dilators, clip applicators, retractors, contractors, excavators, needle holders, suction tubes, coagulation electrodes, electroencephalographic depth electrodes, rib and sternum spreaders, bipolar probes, and rib shears. Alternatively, such article(s) may comprises cutleries and kitchen utensils, such as knives, forks, scissors, peelers, parers, slicers, spatulas, and cleavers, or laboratory apparatus(es), such as containers, filtration devices, centrifuges, spectrophotometers, and fluorometers, or veterinary devices, such as clamps, forceps, knives, saws, probes, and electronic stun equipment.

When the locus to be treated comprises article(s), the proteolytic enzyme used in step (b) is preferably provided in a solution form for purpose of cleansing and sterilizing such articles. While keratinases are used as the proteolytic enzyme, such enzyme solution is preferably characterized by a low effective concentration within the range of from about 0.2 g/L to about 1.0 g/L.

A further aspect of the present invention relates to a method of enhancing degradability of an infectious prion protein by proteolytic enzymatic degradation treatment, including (a) heating the prion protein to a temperature below the pyrolytic destruction temperature of the prion protein, followed by (b) enzymatic degradation treatment of the prion protein.

A still further aspect of the present invention relates to a method of removing infective prion protein from bovine tissue containing or contaminated with same, the method including (a) cooking the bovine tissue at a temperature in a range of from about 100°C to about 150°C, followed by (b) exposing the bovine tissue to a proteolytic enzyme at a temperature in a range of from about 35°C to about 100°C at which the proteolytic enzyme is thermally stable and proteolytically effective to at least partially destroy the infective prion protein associated with the bovine tissue. The cooking is preferably conducted for a time of from about 5 minutes to about 5 hours, and the proteolytic enzyme used in step (b) preferably comprises *Bacillus licheniformis* PWD-1 keratinase.

Thermally stable proteolytic enzymes are particularly useful for the practice of the present invention. Therefore, one specific aspect of the present invention relates to a method of at least partially degrading infectious prion protein in tissue containing or contaminated with same, by steps including heating the tissue and simultaneously exposing same to a thermal stable proteolytic enzyme, at sufficient temperature and for sufficient time to at least partially degrade the infectious prion protein; another specific aspect of the present invention relates to a tissue composition comprising tissue containing or contaminated with an infectious prion protein and a proteolytic enzyme that is thermally stable in a temperature range of from about 35°C to about 100°C. Preferably, such thermally stable proteolytic enzyme is a thermotolerant protease, which can be used to treat animal meat product or by-product under sufficient temperature and for sufficient time to destroy the BSE-mediating infectious prion protein therein.

Yet another aspect of the present invention relates to a method of treatment of tissue for reduction of infective prion protein therein. The method comprises the steps of:

(a) heating the tissue to a sufficient temperature and for sufficient time to enhance the proteolytic degradability of infective prion protein associated with the tissue; and

(b) exposing the heated tissue to a proteolytic enzyme that is effective for at least partial reduction of infective prion protein associated with such tissue.

A further aspect of the present invention relates to a method of disinfecting article(s) that are susceptible to contamination by infectious prion protein, the method comprising the steps of:

(a) heating said article(s) to a sufficient temperature and for sufficient time to enhance the proteolytic susceptibility of infective prion protein associated with said article(s); and

(b) exposing the heated article(s) to a proteolytic enzyme that is effective for at least partial reduction of the infective prion protein associated with said article(s).

In a further aspect, the invention relates to a method of removing infective prion protein from a surgical instrument contaminated with same, the method including (a) heating the surgical instrument at a temperature in a range of from about 100°C to about 150°C, e.g., for a time of from about 5 minutes to about 5 hours, followed by (b) exposing the heated surgical instrument to a proteolytic enzyme at a temperature in a range of from about 35°C to about 100°C at which the proteolytic enzyme is thermally stable and proteolytically effective to at least partially destroy the infective prion protein contaminating the surgical instrument.

A still further aspect of the invention relates to a cleansing composition for disinfecting articles that are susceptible to contamination by infectious prion protein, said composition comprising:

(i) one or more proteolytic protein(s) selected from the group consisting of keratinase enzymes, proteinase K, trypsins, chymotrypsins, pepsins, chymosins, cathepsins, subtilisins, elastases, collagenases, endopeptidases, peptidases, oligopeptidease, thermolysins, bacillolysin, mycilysins, carboxypeptidases, leucyl aminopeptidases, aminopeptidases, extremthermophilic proteases, carbonyl hydrolase, papain, pancreatin, streptokinase, streptodornase, ficin, carboxypeptidase, chymopapain, and bromelin; and

(ii) a solvent.

Preferably, the cleansing composition of the present invention comprises keratinase enzymes in the concentration range of from about 0.2 g/L to 1.0 g/L. Various solvents can be used for the purpose of practicing the present invention, such as distilled water, buffer solution, detergent solution, alcohol, or any other inorganic or organic solvent commonly used in enzymatic detergents, which can be readily determined by a person ordinarily skilled in the art without undue experimentation. More preferably, the cleansing composition further comprises one or more chemical additives for enhancing the disinfection/sterilization results, which include but are not limited to: surfactants, builders, boosters, fillers, and other auxiliaries.

Other aspects, features and embodiments of the invention will be more fully apparent from the ensuing disclosure and appended claims.

BRIEF DESCRIPTION OF THE DRAWING

Figures 1 and 2 illustrate gel electrophoresis/Western blot results on SDS-PAGE gel, evidencing the efficacy of the method of the invention for destruction of infectious prion protein.

DETAILED DESCRIPTION AND PREFERRED EMBODIMENTS OF THE INVENTION

Relative to the present invention and its features, aspects and embodiments as more fully described hereinafter, the disclosures of the technical literature cited in the Background of the Invention section hereof, as well as the following patents and technical literature articles, are hereby incorporated herein by reference in their respective entireties:

U.S. Patent Nos.: 4,908,220; 4,959,311; 5,063,161; 5,171,682; 5,186,961; and 5,712,147;

Deslys, J.P., "Screening slaughtered cattle for BSE," Nature, Vol. 409, pp. 476-477, January 25, 2001; and

Cohen, F.E., "Protein Misfolding and Prion Diseases," J. Mol. Biol. (1999), Vol. 293, pp. 313-320.

The present invention is based on the use of proteolytic enzymes for degradation of infectious prion protein in tissue, and alternatively, for disinfection or sterilization of prion-contaminated articles such as surgical instruments, cutleries and kitchen utensils, veterinary tools, and laboratory tools.

The efficacy of the process of the present invention for degradation of the infectious prion protein is wholly unexpected since high temperature exposure (e.g., at 200°C) of infectious prion proteins alone does not alter their pathogenic character; additionally, conventional proteolytic enzymes such as proteinase K that fully digest non-infectious PrP^{c} do not destroy the corresponding infectious isoform. It therefore is highly surprising that temperatures well below the incineration temperatures heretofore necessary for destruction of infectious PrP^{Sc} can be employed for enzymatic treatment, to totally eliminate infectious PrP^{Sc} from tissue containing or contaminated with same.

As used herein, the term elevated temperature means temperature of at least 35°C. The term proteolytic susceptibility means the ability of an infective prion protein to be enzymatically degraded to a non-infective product.

The treatment of a locus, such as tissues or articles, for reduction of infective prion protein associated therewith, can be carried out by various techniques as hereinafter described.

For example, the tissue or the article (which may contain, be contaminated with, or be suspected to contain or be contaminated with, infectious prion protein) in one embodiment of the invention is heated to a sufficient temperature and for sufficient time to enhance the proteolytic susceptibility of infective prion protein that may be present, in conjunction with exposure of the tissue or article to a proteolytic enzyme that is effective to at least partially destroy any infective prion protein that is present.

Such treatment can be carried out in a two-step sequence, including an initial step of heating the tissue or article to a first higher elevated temperature and then exposing the heated tissue or article to the

enzymatic agent at a second lower elevated temperature, for proteolytic degradation of the infectious prion protein.

In such two-step process, the tissue or article can be thermally treated at a first higher elevated temperature, and then cooled to second lower elevated temperature, e.g., by radiative heat loss from the tissue or article, convective cooling of the tissue or article, or in other appropriate manner, so that the tissue or article is at a suitable temperature when it is inoculated with or otherwise exposed to the proteolytic enzyme in the second enzymatic treatment step.

In the second step of such two-step process, the tissue or article is exposed to a proteolytic enzyme that is effective to at least partially destroy the infective protein prion associated with the tissue or article.

The method can therefore be carried out in various embodiments in which proteolytic susceptibility of infective prion protein associated with the tissue or article is enhanced by heating of the tissue or article to an elevated temperature for subsequent proteolytic enzyme treatment. The elevated temperature in the heating step may be any suitable temperature, e.g., at least 35°C, at least 40°C, at least 60°C, at least 75°C, and/or no more than 150°C (or other lower temperature, as desired), with one illustrative specific temperature range being from about 100°C to about 150°C, and more preferably from about 125°C to about 140°C.

Alternatively, the prion protein destruction treatment of the invention may be carried out in a single-step procedure, in instances where the proteolytic enzyme is stable and effective (to remove the infectious prion protein) at the corresponding temperature used in the treatment process, so that no initial heating step is required.

In the single step method, the animal tissue or the article, together with the proteolytic enzyme, are heated to a suitable elevated temperature for the enzymatic degradation of the infectious prion protein to occur.

For example, the method of at least partially degrading infectious prion protein in tissue or article containing or contaminated with same, can be carried out by heating the tissue or article and simultaneously exposing same to a thermal stable proteolytic enzyme, at sufficient temperature and for sufficient time to at least partially degrade the infectious prion protein.

Regardless of the specific sequence of method steps employed in the practice of the invention, the tissue or article is exposed to a proteolytic enzyme (in the second step of the two-step method, or in the enzymatic degradation step of the single-step method) that is effective to at least partially destroy the infective protein prion associated therewith.

The enzymatic degradation step can be carried out at any suitable temperature in the practice of the invention, e.g., at a temperature above about 35°C, above about 40°C, or above about 50°C, depending on the thermostable character of the proteolytic enzyme employed.

By way of illustrative examples, the enzymatic degradation step can be conducted at a temperature in a range of from about 35°C to about 100°C, from about 40°C to about 100°C, from about 50°C to about 100°C, from about 40°C to about 75°C, or from about 50°C to about 60°C, depending on the proteolytic stability and enzymatic activity of the specific proteolytic enzyme that is employed.

In the enzymatic degradation step, the proteolytic enzyme at least partially, and preferably completely, destroys the infective protein prion that is in or otherwise associated with the tissue or article to be treated.

It will be recognized that any of a wide variety of proteases may be employed in the practice of the invention, and that the choice of specific proteolytic enzyme will affect the choice of temperature that is used to carry out the proteolytic degradation, as well as the choice of any elevated temperature treatment of the tissue or article before its exposure to the proteolytic enzyme.

Specific temperature treatment conditions for the enzymatic treatment, as well as the temperature conditions necessary or desirable for any elevated temperature initial treatment step(s) that precede such enzymatic treatment, can be readily empirically determined without undue experimentation, within the skill of the art.

Proteolytic enzymes usefully employed in the practice of the invention include enzymes that are enzymatically active and effective at the conditions of their use. For elevated temperature enzymatic treatment, the proteolytic enzyme is suitably thermostable at the conditions of use.

In this respect, proteolytic enzymes of widely varying thermostable character are known. For example, various proteolytic enzymes employed in specific embodiments of the invention may be thermostable up to 35°C, 40°C, 50°C, 60°C, or even 100°C.

The proteolytic enzyme may be of any suitable type, and may comprise a single enzymatic species, or alternatively a mixture of enzymes. The enzyme may be used in a purified and concentrated form, or alternatively in a diluted form. It is preferred that the enzyme is dissolved in a solvent to form an enzyme solution with a concentration of from about 0.2 g/L to about 1.0 g/L.

Illustrative proteolytic enzymes in the broad practice of the present invention include, without limitation, keratinase enzymes, proteinase K, trypsins, chymotrypsins, pepsins, chymosins, cathepsins, subtilisins, elastases, collagenases, endopeptidases, peptidases, oligopeptidease, thermolysins, bacillolysin, mycilysins, carboxypeptidases, leucyl aminopeptidases, aminopeptidases, extremthermophilic proteases, carbonyl hydrolase, papain, pancreatin, streptokinase, streptodornase, ficin, carboxypeptidase, chymopapain, and bromelin.

Preferred enzyme species include keratinase enzymes. A particularly preferred keratinase comprises *Bacillus licheniformis* PWD-1 keratinase. Proteolytic enzyme species useful in the practice of the invention include active fragments of proteolytic enzymes, e.g., an active fragment of a keratinase

enzyme, such as the *Bacillus licheniformis* PWD-1 keratinase. When keratinase enzymes are used in the present invention, the effective concentration required for the enzyme solution is significantly lower than that of the conventional enzyme detergents or disinfectants. Moreover, keratinase enzymes are characterized by an optimal active temperature range of from about 50°C to about 65°C at pH value of from about 6.0 to about 9.5, which is significantly higher than that of most conventional enzyme detergents. Therefore, the cleansing temperature of the process of the present invention can be significantly increased, which is more efficient for enhancing the proteolytic susceptibility of the infective prion protein associated with the surgical instruments.

In the method of the invention for treating tissues, the tissue being treated may be of any suitable type, including mammalian as well as non-mammalian animal tissues, and even plant tissues that actually or potentially contain infectious prion proteins. Mammalian tissues can include human as well as non-human mammalian tissues.

In a specific aspect, the tissues treatable in the method of the invention include, without limitation, bovine tissue, ovine tissue, simian tissue, and human tissue, and include various tissue types, e.g., brain, pituitary, intestine, lung, heart, kidney, and/or spleen tissue. In one aspect, the method of the invention is employed to treat nervous system tissue, which may be central nervous system tissue and/or peripheral nervous system tissue.

The method of enzymatically removing infectious prion proteins from tissue or disinfecting/sterilizing article such as surgical instrument or the like, in accordance with the present invention, can further include the step(s) of testing the tissue or article to verify destruction of infective prion protein associated therewith, after proteolytic enzymatic treatment has been concluded. The testing of the tissue or article for infectious prion protein may be carried out in any suitable manner and with any suitable testing technique or methodology, e.g., by subjecting the tissue or article to a Western blot test, sandwich immunoassay test, ELISA test, fluoroimmunoassay test, capillary immuno-electrophoresis test,

plasminogen binding test, or other suitable test that is efficacious for determining the presence or absence of infectious prion protein in the tissue being treated.

The enzymatic treatment method of the invention can be carried out in any suitable manner, with any appropriate sequence of processing steps.

For example, in one embodiment, the tissue or article to be treated is subjected to initial non-enzymatic thermal treatment as necessary or desired, followed by enzymatic treatment for destruction of infective or contaminative prion protein, followed by rinsing and non-enzymatic treatment, testing of the tissue or article treated, and/or further thermal/enzymatic treatment (e.g., in an alternating and repetitive cycle of non-enzymatic thermal treatment, and enzymatic elevated temperature treatment), as required, if the post-treatment testing shows incomplete removal of the infectious prion protein from the tissue or article.

In another embodiment, infectious prion protein is at least partially degraded in tissue or article containing or contaminated with same, by steps including heating the tissue or article and simultaneously exposing same to a thermal stable proteolytic enzyme, at sufficient temperature and for sufficient time to at least partially degrade the infectious prion protein. The treated tissue or article then may be subjected to testing to characterize the removal of the infectious prion protein.

Further, the method of the invention may include an initial determination of the presence of infectious prion protein associated with the tissue or article being treated, before any thermal/enzymatic treatment, so that treatment is only applied to tissue or article that is established by such determination as containing infectious prion protein.

Alternatively, the thermal/enzymatic treatment may be administered to tissue or article that may potentially contain or be contaminated with, but is not definitively ascertained beforehand to verify the presence of, infective prion protein, followed by testing of the treatment product for determination of the presence or absence of any infectious prion protein associated therewith.

The method of the invention is efficaciously applied to destroy infectious prion proteins in meat and meat by-products that are susceptible to containing or being contaminated with infectious prion proteins mediating TSEs such as BSE.

As such, the method of the invention provides a reliable approach to the treatment of bovine and other animal products and by-products that then can be further processed in food processing and/or rendering operations, rather than being incinerated to avoid transmission of BSE and other infectious prion protein diseases.

The invention thus contemplates in one embodiment a method of processing animal products and by-products, in which the prion protein is initially non-enzymatically thermally treated, e.g., by heating to a temperature that is below the pyrolytic destruction temperature (>>200°C) of the infectious prion protein (at which incineration is generally conducted), followed by enzymatic degradation of the infectious prion protein.

In a specific illustrative embodiment of the invention, infective prion protein is removed from bovine tissue containing same, by cooking the bovine tissue at temperature in a range of from about 100°C to about 150°C, e.g., for a time of from about 5 minutes to about 5 hours, followed by exposing the bovine tissue to a proteolytic enzyme at temperature in a range of from about 35°C to about 100°C at which the proteolytic enzyme is thermally stable and proteolytically effective to destroy the infective prion protein in the bovine tissue.

The cooking treatment can be carried out in a suitable chamber or vessel in which elevated temperature conditions are appropriately maintained, optionally with control of pressure to provide a desired atmospheric, sub-atmospheric, or superatmospheric pressure in the cooking operation.

After cooking, the bovine tissue is subjected to proteolytic enzymatic treatment with *Bacillus licheniformis* PWD-1 keratinase to destroy all infectious prion protein therein. Following the thermal/enzymatic treatment, the tissue may be processed in any suitable manner.

For example, such bovine tissue, e.g., after testing or assay verification of the complete destruction of infectious prion protein, can be processed to yield an animal feed ingredient (e.g., meat bone meal) or feed supplement.

In one particularly preferred process embodiment, the cooking step is carried out in a first elevated temperature range of from about 125°C to about 150°C, and enzymatic treatment of the bovine tissue thereafter is carried out using *Bacillus licheniformis* PWD-1 keratinase, in a second elevated temperature range of from about 40°C to about 60°C.

The method of the present invention enables the utilization of rendered bovine meat by-products that would otherwise (in suspicion or verification of the presence of infectious prion proteins), require incineration and disposal.

The inventive method thereby achieves a substantial advance in the art, permitting nutritional use of material that would otherwise, in the absence of treatment, constitute a biological hazard. The inventive method concurrently avoids the costs and infrastructure requirements for incineration and disposal of infected or contaminated animal tissue.

The invention embodies a simple methodology for removing infectious prion protein, e.g., BSE-mediating prion protein, from tissue, by exposing the tissue to a proteolytic enzyme that is thermally stable, at sufficient temperature and for sufficient time to at least partially clear the infectious prion protein from the tissue.

The method of the invention is broadly applicable to the destruction of prion protein that is infectious for transmissible spongiform encephalopathy (TSE) and/or for other prion protein-mediated diseases, including, without limitation, bovine spongiform encephalopathy (BSE) and sheep scrapie.

The method of the invention has applicability to processing animal meat for human food and processing of animal by-product for animal feed or an animal feed ingredient.

The present invention in one compositional aspect comprehends a tissue composition including (i) tissue, e.g., bovine tissue, containing an infectious prion protein, such as prion protein mediating BSE, and (ii) a proteolytic enzyme, e.g., *Bacillus licheniformis* PWD-1 keratinase, that is thermally stable in the temperature range employed for enzymatic treatment, e.g., from about 40°C to about 60°C.

Such tissue composition may be at elevated temperature. The composition is enzymatically reactive at suitable elevated temperature to produce a product composition including the proteolytic enzyme and the treated tissue free of infectious prion protein.

While the invention has been illustratively described hereinabove primarily in application to the treatment of infected or contaminated animal tissue in a harvested state, such as rendered animal parts for the subsequent production of animal feed ingredients, the invention also comprehends the application of proteolytic enzymes for *in vivo* treatment of prionic diseases.

In one such embodiment, a therapeutic composition is provided for *in vivo* prionic disease treatment, including as an active ingredient a proteolytic enzyme that is combatingly effective against the prionic disease.

Another embodiment includes a therapeutic composition containing a keratinase in combination with a non-infectious prion protein (e.g., PrP^{c}, or a PrP^{Sc} that has been modified to render same non-infectious, or a non-infectious fragment of PrP^{Sc}) serving as a molecular recognition protein for the infectious prion protein *in vivo*.

Still other therapeutic compositions are contemplated that include a keratinase or other proteolytic enzyme in vectorized constructs or combinations.

Another illustrative therapeutic composition includes a pyrogenic agent, such as a non-toxic modified endotoxin analog, in combination with a proteolytic enzyme that is combatingly effective against the prionic disease upon induction of pyrogenesis *in vivo*.

The invention also comprehends *in vivo* therapeutic compositions, comprising a polynucleotide sequence including a first region coding for the production of the proteolytic enzyme or active fragment thereof, and a second region coding for a pyrogenic peptide, as part of a recombinant polynucleotide expression vector. Following transfection, the *in vivo* expression of the thermotolerant proteolytic enzyme or active fragment thereof, and the pyrogenic peptide, effect infectious prion protein-combating action.

As a still further example, the therapeutic composition may in application to transmissible spongiform encephalopathy be formulated with a blood-brain barrier traversing agent, such as an amphiphilic drug-oligomer conjugate capable of traversing the blood-brain barrier. Such composition can comprise the therapeutic compound, e.g., keratinase or other proteolytic enzyme, or an active fragment thereof, conjugated to an oligomer, wherein the oligomer comprises a lipophilic moiety coupled to a hydrophilic moiety. Oligomers useful for formulating such therapeutic compositions are more fully described in International Publication WO 00/09073 published February 24, 2000.

Another important application of the method of the present invention is disinfection and/or sterilization of articles, such as surgical instruments, cutleries, kitchen utensils, laboratory apparatus, and veterinary tools. Such method is broadly applicable to the destruction of prion protein contaminates associated with:

(a) surgical instruments, such as clamps, forceps, scissors, knives, cables, punches, tweezers, cannulae, calipers, carvers, curettes, scalers, dilators, clip applicators, retractors, contractors, excavators, needle

holders, suction tubes, trocars, coagulation electrodes, electroencephalographic depth electrodes, rib and sternum spreaders, bipolar probes, rib shears, etc.;

(b) cutlery and kitchen utensils, such as knives, forks, scissors, peelers, parers, slicers, spatulas, and cleavers; and

(c) laboratory tools, such as filtration devices, centrifuges, spectrophotometers, fluorometers, and various containers and

(d) veterinary tools and devices, such as clamps, forceps, knives, saws, probes and electronic stun equipment.

The above list is only illustrative of several application of the present invention, and it should not be construed in any manner as to limit the scope of the present invention.

The following table shows a disinfection/sterilization cycle according to one embodiment of the present invention:

TABLE I

Steps	Temperature	Time
Pre-Wash (cold water)	Room Temp.	2-5 minutes
Heating	35-100°C	20-40 minutes
Cooling	34-51°C	2-10 minutes
Enzyme Wash	34-51°C	20-120 minutes
Sonication	34-51°C	5 minutes
Detergent Wash	51-57°C	2-5 minutes
Rinse and Dry	Room Temp.	5 minutes
Autoclave Sterilization	200-500°C	---

In another embodiment, a thermally stable proteolytic enzyme is used for such disinfection/sterilization process, so that the heating and enzymatic wash steps can be conducted simultaneously, at sufficient temperature and for sufficient time for complete destruction of the infection prion protein and sterilization of the treated articles.

The present invention in one compositional aspect comprehends a cleansing composition including (i) a proteolytic enzyme, e.g., *Bacillus licheniformis* PWD-1 keratinase, that is thermally stable in the temperature range employed for enzymatic treatment, e.g., from about 40°C to about 60°C; and (ii) a solvent.

Any solvent that is suitable for use with enzymatic detergents can be employed in the composition of the present invention. Distilled water is a preferred solvent, in light of its biological compatibility and low costs. Other conventional inorganic and organic solvents, such as alcohol, buffer solution, detergent solution, can also be used for purpose of practicing the present invention, and a person ordinarily skilled in the art can readily select solvents that are compatible with specific enzymes used. Chemical additives that are conventionally employed can also be introduced into the cleansing composition of the present invention, which include but are not limited to surfactants, builders, boosters, fillers, and other auxiliaries.

The cleansing composition of the present invention retains its effectiveness for destructing infectious prion protein even at a very low concentration, for example, of less than 0.3 g/L. When keratinase enzyme is employed in such cleansing composition, the enzyme concentration of such composition is preferably within the range of from about 0.2 g/L to about 1.0 g/L.

The cleansing composition of the present invention is enzymatically reactive at elevated temperature, and it therefore may be used at elevated temperature for complete destruction of infectious prion protein associated with surgical instruments, cutleries, kitchen utensils, veterinary tools, and laboratory tools.

The features and advantages of the invention are more fully shown with reference to the following illustrative example.

EXAMPLE 1

A feather-degrading bacterium, *Bacillus licheniformis* strain PWD-1, isolated from a thermophilic anaerobic digester for poultry waste (see C.M. Williams and J.C.H. Shih, *J. Appl. Bacteriol.* **67**, 25 (1989); J.C.H. Shih, *Poultry Sci.* **72**, 1617 (1993)) was the source of the keratinase enzyme (see X. Lin, C.G. Lee, E.S. Casale, and J.C.H. Shih, *Appl. Environ. Microbiol.* **58**, 3271 (1992)) employed in this example.

The gene encoding this keratinase enzyme (see X. Lin, D.W. Kelemen, E.S. Miller and J.C.H. Shih, *Appl. Env. Microbiol.* **61**, 1469 (1995)) has been isolated and sequenced, and scale-up fermentation production of this enzyme has also been accomplished (see J.J. Wang and J.C.H. Shih, *J. Ind. Microb. Biotech.* **22**, 608 (1999)). This enzyme is a serine protease.

Crude and purified preparations of this keratinase were produced as previously described (see X. Lin, C.G. Lee, E.S. Casale, and J.C.H. Shih, *Appl. Environ. Microbiol.* **58**, 3271 (1992) and J.J. Wang and J.C.H. Shih, *J. Ind. Microb. Biotech.* **22**, 608 (1999)) and obtained from the Fermentation Facility at North Carolina State University, Raleigh, North Carolina (NCSU). The test for the effect of keratinase on PrP was carried out at Institute of Animal Science and Health at Lelystad (ID-Lelystad), The Netherlands.

Purified keratinase was compared with other proteases, including elastase, collagenase, proteinase K and trypsin (all from Sigma chemical Co.) in reacting with various kinds of substrates. Hydrolysis of keratin, elastin and collagen were measured by ninhydrin color reaction (A_{450}) of increased free amino groups (see X. Lin, C.G. Lee, E.S. Casale, and J.C.H. Shih, *Appl. Environ. Microbiol.* **58**, 3271 (1992)). Free leucine was used as the standard to calculate the equivalent free amino groups. Casein hydrolysis was measured by the increased A_{280} in the supernatant (see Price and Johnson, 1989). The results are presented in Table

1 below. For each given substrate, relative activities of all proteases were determined. Cumulative relative activity (CRA) demonstrated that the keratinase has a wide range of substrates and possesses high activity.

TABLE 1. Relative specific activities of proteases against different substrates[a]

Substrate	Keratinase	Elastase	Collagenase	Proteinase K	Trypsin
Keratin[b]	**1.00**	0.29	0.00	0.36	0.09
Elastin[b]	2.52	**1.00**	0.43	0.57	0.61
Collagen[b]	2.58	1.15	**1.00**	0.70	0.38
Casein[c]	1.28	0.80	0.02	**1.00**	0.40
CRA[d]	7.38	3.24	1.45	2.63	1.48

[a]All enzyme activities were measured at their individual optimum conditions and compared.

[b]Proteolysis measured by ninhydrin reaction (Lin et al., 1992).

[c]Proteolysis measured by increased soluble A_{280} (Price and Johnson, 1989).

[d]Cumulative Relative Activity.

The test of the effect of keratinase on pathogenic PrP was carried out in an Isolation Facility in the Laboratory of Molecular Recognition, ID-Lelystad. The European Union-validated procedure of Prionics Check (Prionics AG, Zurich) was used to detect pathogenic PrP. The Prionics Check procedure is based on the Western blot technique and employs 6H4 monoclonal antibody to visualize the specific form of PrP (Prionics AG, Test for the Detection of BSE-prions in Cattle, Practical Product Information, Zurich (2000)).

In order to mimic a meat bone meal process, modifications were made from the original procedure. First, proteinase K in the standard procedure was replaced by keratinase. A crude preparation of keratinase was used. Second, the effect of a pre-cooking of the BSE tissue was tested. The homogenized tissue was cooked at 115°C for 40 minutes with a Vulcain pressure-cooker. Third, an anti-oxidant, sodium sulfite (Na_2SO_3), also was tested. The rest of the procedure was the same as described in the manufacturer's Practical Product Information (Prionics AG, Test for the Detection of BSE-prions in Cattle, Practical Product Information, Zurich (2000)).

The following protocol was employed. One g of BSE-positive brain tissue was mixed and homogenized with 9 ml of Prionics buffer. Half of the aliquot, 5 ml, was added with Na_2SO_3 to give a final concentration 0.1% and the other half, without Na_2SO_3. The aliquots were distributed 4 x 2.0 ml into autoclavable Falcon tubes. An additional 1.0 ml was for used for positive control, treated by the standard Prionics procedure, and another 1.0 ml, for no keratinase control. Two tubes, with and without Na_2SO_3, were pressure-cooked for 40 min and the other 2 tubes were not cooked. In the wells of the PCR plate, the samples, 150 µl each, were treated by keratinase (150 µg, 1,000 EU/mg) at 50°C for 0-time or 4 hrs. Keratinase was pre-dissolved in phosphate buffer, 0.05 M, pH 7.5. The reaction was stopped by the addition of Prionics Pefabloc, an inhibitor of serine protease. At the end of enzymatic incubation, 10 µl of each sample mixture was loaded onto SDS-PAGE gel, and the Prionics procedure of electrophoresis, Western blot, and immuno-chemiluminescence detection was followed.

The results of this experiment are shown in Figure 1 (effect of keratinase degradation on BSE-prion protein), wherein Lanes 1-17 are as follows:

Lane 1: buffer alone.

Lane 2: BSE-brain tissue as tested.

Lane 3: Pre-cooked with Na_2SO_3, keratinase stopped at 0-time.

Lane 4: Same as Lane 3, except keratinase digestion for 4 hr.

Lane 5: Pre-cooked without Na_2SO_3, keratinase stopped at 0-time.

Lane 6: Same as Lane 5, keratinase digestion for 4 hr.

Lane 7: Without pre-cooking, with Na_2SO_3, keratinase 0-time.

Lane 8: Same as Lane 7, except keratinase digestion 4 hr.

Lane 9: Without pre-cooking, without Na_2SO_3, keratinase 0-time.

Lane 10: Same as Lane 9, except keratinase digestion 4 hr.

Lane 11: Without pre-cooking, with Na_2SO_3, no keratinase.

Lane 12: Same as Lane 11, except incubation 4 hr (Note: keratinase was accidentally added).

Lane 13: Purified scrapie PrP with Na_2SO_3, keratinase stopped at 0-time.

Lane 14: Same as Lane 13, except keratinase digestion 4 hr.

Lane 15: Purified scrapie PrP with Na_2SO_3, without keratinase.

Lane 16: Same as Lane 15, except incubation 4 hr.

Lane 17: PrP standard.

As shown in Fig. 1, the digestive effect of keratinase on infectious PrP is evident, particularly when the samples were precooked at 115°C for 40 min (Lanes 3-6). Without precooking (Lanes 7-10), the keratinase was less effective, but keratinase still degraded more than half of the infectious PrP positive material. On purified sheep scrapie PrP, keratinase was found to be active as well (Lanes 13-16). The

presence of Na_2SO_3 did not appear to make much difference (Lanes 15-16) either alone or with keratinase. The Lane 12 sample was accidentally added with keratinase and therefore was positive.

EXAMPLE 2

Experiments have been conducted to further evidence the efficacy of the invention, according to the following experimental procedure:

PWD-1 keratinase, a serine protease derived from Bacillus licheniformis strain PWD-1, was used to effect degradation of PrP^{Sc} in infected bovine and ovine brain stem tissues. The Prionics Check test (Prionics AG, Zurich, Switzerland) was used to determine PrP^{Sc} in the samples, following precooking of the tissue sample, and digestion of the tissue sample with purified PWD-1 keratinase as the digestive enzyme.

In each instance, 1 g of BSE-positive brain stem tissue was mixed and homogenized with 9 ml. of Prionics homogenization buffer, followed by pressure-cooking at 115°C for 40 minutes with a Vulcan pressure cooker. Into a 96-well plate, 150 µl of each sample was added and treated with PWD-1 keratinase pre-dissolved in phosphate buffer, 0.05 M, pH 7.5. An enzyme concentration of 250 µg/ml was used. Enzymatic digestion was carried out at 50°C for 60 min. The reaction was stopped by the addition of 15 µl Pefabloc®, an inhibitor of serine protease. Time-zero samples were treated by first adding the inhibitor before the addition of the PWD-1 keratinase. At the end of the enzymatic incubation, 10 µl of each sample was loaded onto the SDS-PAGE gel. Electrophoresis, Western blotting and immuno-chemiluminescence detection were conducted as outlined in the Prionics Check kit.

Brain stem tissue samples, 3 BSE-positive (samples B1, B2, B3), 1 negative (bovine sample N), 2 scrapie positive (samples S1 and S2) and 1 negative (ovine sample N) were tested by the purified PWD-1 keratinase, against corresponding controls.

Figure 2 shows the results, with the left-hand panel ("Keratinase-treated) being the result of keratinase digestion, and the right-hand panel ("Control") being the result of the standard Prionic Check. The keratinase was able to hydrolyze all PrP^{Sc} tested, BSE (lanes 3-5) as well as scrapie (lanes 8 and 9).

Figure 2 hereof evidence the complete destruction of infectious prion protein in bovine tissue samples containing same (samples B1, B2, B3) and in ovine samples containing same (samples S1 and S2), when such samples were treated by combined thermal and enzymatic treatment in accordance with the Invention.

These results demonstrate the versatility of the keratinase in degrading all types of proteins tested. In the test of the efficacy of the keratinase on BSE PrP, the results were positive, especially when the BSE brain tissue samples were pre-cooked. This is the first experiment to demonstrate that pathogenic PrP is degradable by an enzyme.

Pressure cooking at temperatures on the order of 125°C is a routine step in processing animal by-products into meat bone meal. Post-cooking treatment with keratinase in accordance with the invention, for destruction of infectious PrP, provides an effective method to control the spread of BSE. The keratinase-treated meat bone meal is readily tested to verify the absence of PrP, so that the meat bone meal can be recycled for feed use.

The method of the invention thus provides a simple and useful enzymatic treatment for animal product and by-product processing.

While the invention has been described herein with reference to various illustrative features, aspects, and embodiments, it will be appreciated that the utility of the invention is not thus limited, but rather extends

to and encompasses other variations, modifications and other embodiments, as will readily suggest themselves to those of ordinary skill in the art.

Accordingly, the invention is to be broadly interpreted and construed as including such other variations, modifications and other embodiments, within the spririt and scope of the invention as hereinafter claimed.

INDUSTRIAL UTILITY

The method and composition of the present invention are useful for destruction of infectious prion proteins, and are applicable to the treatment of biological materials, e.g., animal tissue containing or contaminated with infectious prion proteins. The invention enables processing of biological materials, which would otherwise (in suspicion or verification of the presence of infectious prion proteins) require incineration and disposal, into useful and safe animal feeds or other nutritional end products. The method and composition of the present invention are also useful for disinfection and/or sterilization of articles, such as surgical instruments, cutleries, kitchen utensils, laboratory apparatus, and veterinary tools, and effectively prevent cross-contamination and propagation of infective prion protein caused by reuse of such articles.

CLAIMS

1. A method of treatment for reduction of infective prion protein at a locus contaminated or suspected of being contaminated with infective prion protein, the method comprising the steps of:

   (a) heating the locus to a sufficient temperature and for sufficient time to enhance the proteolytic susceptibility of infective prion protein at the locus; and

   (b) exposing the heated locus to a proteolytic enzyme that is effective for at least partial reduction of the infective protein prion at such locus.

2. The method of claim 1, wherein the temperature in step (a) comprises a temperature not exceeding about 150°C.

3. The method of claim 1, wherein the temperature in step (a) comprises a temperature in a range of from about 100°C to about 150°C.

4. The method of claim 1, wherein the temperature in step (a) comprises a temperature in a range of from about 125°C to about 140°C.

5. The method of claim 1, wherein step (b) is carried out at a temperature in a range of from about 35°C to about 100°C.

6. The method of claim 1, wherein step (b) is carried out at a temperature above about 40°C.

7. The method of claim 1, wherein step (b) is carried out at a temperature above about 50°C.

8. The method of claim 1, wherein step (b) is conducted at a temperature that is lower than that of step (a).

9. The method of claim 1, wherein step (b) is carried out at a temperature in a range of from about 40°C to about 75°C.

10. The method of claim 1, wherein step (b) is carried out at a temperature in a range of from about 50°C to about 60°C.

11. The method of claim 1, wherein the proteolytic enzyme comprises an enzyme selected from the group consisting of keratinase enzymes, proteinase K, chymotrypsins, pepsins, chymosins, cathepsins, subtilisins, elastases, collagenases, endopeptidases, peptidases, oligopeptidease, thermolysins, bacillolysin, mycilysins, carboxypeptidases, leucyl aminopeptidases, aminopeptidases, extremthermophilic proteases, carbonyl hydrolase, papain, pancreatin, streptokinase, streptodornase, ficin, carboxypeptidase, chymopapain, and bromelin.

12. The method of claim 1, wherein the proteolytic enzyme comprises a keratinase enzyme and/or an active fragment thereof.

13. The method of claim 1, wherein the proteolytic enzyme comprises a Bacillus licheniformis PWD-1 enzyme and/or an active fragment thereof.

14. The method of claim 1, further comprising a step of (c) testing the locus to verify reduction of infective prion protein therein.

15. The method of claim 14, wherein the testing step (c) comprises subjecting the locus to a test selected from the group consisting of Western blot tests, sandwich immunoassay tests, ELISA

tests, fluoroimmunoassay tests, capillary immuno-electrophoresis tests, and plasminogen binding tests.

16. The method of claim 14, wherein the testing step (c) comprises subjecting the locus to a Western blot test.

17. The method of claim 1, wherein said locus comprises tissue containing or contaminated by infective prion protein therein.

18. The method of claim 17, wherein said tissue comprises mammalian tissue.

19. The method of claim 17, wherein said tissue comprises nervous system tissue.

20. The method of claim 17, wherein said tissue comprises bovine tissue.

21. The method of claim 17, wherein the tissue comprises BSE-infected tissue.

22. The method of claim 17, wherein said tissue comprises ovine tissue.

23. The method of claim 17, wherein said tissue comprises scrapie-infected tissue.

24. The method of claim 17, wherein said tissue is selected from the group consisting of brain, pituitary, intestine, lung, heart, kidney, and spleen tissues.

25. The method of claim 17, wherein said tissue is from a carrier animal for the infective prion protein.

26. The method of claim 1, wherein said locus comprises article(s) that are susceptible to contamination by infectious prion protein.

27. The method of claim 26, wherein said article(s) comprise surgical instrument(s).

28. The method of claim 27, wherein said surgical instrument(s) are selected from the group consisting of: clamps, forceps, scissors, knives, cables, punches, tweezers, cannulae, calipers, carvers, curettes, scalers, dilators, clip applicators, retractors, contractors, excavators, needle holders, suction tubes, coagulation electrodes, electroencephalographic depth electrodes, rib and sternum spreaders, bipolar probes, and rib shears.

29. The method of claim 26, wherein said article(s) comprise cutleries and kitchen utensils.

30. The method of claim 29, wherein said cutleries and kitchen utensils are selected from the group consisting of: knives, forks, scissors, peelers, parers, slicers, spatulas, and cleavers.

31. The method of claim 26, wherein said article(s) comprise laboratory apparatus(es).

32. The method of claim 31, wherein said laboratory apparatus(es) are selected from the group consisting of: containers, filtration devices, centrifuges, spectrophotometers, and fluorometers.

33. The method of claim 26, wherein said article(s) comprise veterinary devices.

34. The method of claim 33, wherein said veterinary devices are selected from the group consisting of clamps, forceps, knives, saws, probes, and electronic stun equipment.

35. The method of claim 1, wherein said proteolytic enzyme comprises a protease enzyme.

36. The method of claim 35, wherein the protease enzyme comprises a carbonyl hydrolase.

37. The method of claim 36, wherein the carbonyl hydrolase comprises subtilisin.

38. The method of claim 37, wherein the subtilisin comprises a mutant of wild-type Bacillus amyloliquefaciens subtilisin, comprising one or more amino acid substitutions, additions, or deletions.

39. A method of enhancing degradability of an infectious prion protein by proteolytic enzymatic degradation treatment, including (a) heating the prion protein to a temperature below the pyrolytic destruction temperature of the prion protein, followed by (b) enzymatic degradation treatment of the prion protein.

40. A method of removing infective prion protein from bovine tissue containing or contaminated with same, the method including (a) cooking the bovine tissue at a temperature in a range of from about 100°C to about 150°C, followed by (b) exposing the bovine tissue to a proteolytic enzyme at a temperature in a range of from about 35°C to about 100°C at which the proteolytic enzyme is thermally stable and proteolytically effective to at least partially destroy the infective prion protein associated with the bovine tissue.

41. The method of claim 40, wherein said cooking is conducted for a time of from about 5 minutes to about 5 hours.

42. The method of claim 40, wherein the proteolytic enzyme comprises *Bacillus licheniformis* PWD-1 keratinase.

43. A method of degrading an infectious prion protein, comprising (a) heating the infectious prion protein to temperature in a first elevated temperature range, followed by (b) cooling the infectious prion protein to lower elevated temperature in a second elevated temperature range, and (c) exposing the infectious prion protein to a proteolytic enzyme effective at such lower elevated temperature to degrade the infectious prion protein to a benign degradation product.

44. A method of at least partially degrading infectious prion protein in tissue or article containing or contaminated with same, by steps including heating the tissue or article and simultaneously exposing same to a thermally stable proteolytic enzyme, at sufficient temperature and for sufficient time to at least partially degrade the infectious prion protein.

45. A method of processing an animal meat product or by-product to clear BSE-mediating infectious prion protein therefrom, the method comprising treating the animal meat product or by-product with a thermotolerant protease under sufficient temperature and for sufficient time to destroy the BSE-mediating infectious prion protein therein.

46. A tissue composition comprising tissue containing or contaminated with an infectious prion protein and a proteolytic enzyme that is thermally stable in a temperature range of from about 35°C to about 100°C.

47. A method of treatment of tissue for reduction of infective prion protein therein, the method comprising the steps of:

(a) heating the tissue to a sufficient temperature and for sufficient time to enhance the proteolytic susceptibility of infective prion protein in the tissue; and

(b) exposing the heated tissue to a proteolytic enzyme that is effective for at least partial reduction of the infective protein prion in such tissue.

48. A method of disinfecting article(s) that are susceptible to contamination by infectious prion protein, the method comprising the steps of:

(a) heating said article(s) to a sufficient temperature and for sufficient time to enhance the proteolytic susceptibility of infective prion protein associated with said article(s); and

(b) exposing the heated article(s) to a proteolytic enzyme that is effective for at least partial reduction of the infective prion protein associated with said article(s).

49. A method of removing infective prion protein from a surgical instrument contaminated with same, the method including (a) heating the surgical instrument at a temperature in a range of from about 100°C to about 150°C, followed by (b) exposing the heated surgical instrument to a proteolytic enzyme at a temperature in a range of from about 35°C to about 100°C at which the proteolytic enzyme is thermally stable and proteolytically effective to at least partially destroy the infective prion protein contaminating said surgical instrument.

50. A cleansing composition for disinfecting articles that are susceptible to contamination by infectious prion protein, said composition comprising:

(i) one or more proteolytic protein(s) selected from the group consisting of keratinase enzymes, proteinase K, trypsins, chymotrypsins, pepsins, chymosins, cathepsins, subtilisins, elastases, collagenases, endopeptidases, peptidases, oligopeptidease, thermolysins, bacillolysin, mycilysins, carboxypeptidases, leucyl aminopeptidases, aminopeptidases, extremthermophilic proteases, carbonyl hydrolase, papain, pancreatin, streptokinase, streptodornase, ficin, carboxypeptidase, chymopapain, and bromelin; and

(ii) a solvent.

51. The cleansing composition of claim 50, comprising keratinase enzymes.

52. The cleansing composition of claim 51, wherein the concentration of said keratinase enzymes is within the range of from about 0.2 g/L to about 1.0g/L.

53. The cleansing composition of claim 50, wherein the solvent is selected from the group consisting of distilled water, alcohol, buffer solution, and detergent solution.

54. The cleansing composition of claim 50, further comprising one or more chemical additives selected from the group consisting of surfactants, builders, boosters, and fillers.

55. A method of treatment of tissue for reduction of infective prion protein therein, the method comprising the steps of:

(a) heating the tissue to a temperature of at least 40°C and for sufficient time to enhance the proteolytic susceptibility of infective prion protein in the tissue; and

(b) exposing the heated tissue to a proteolytic enzyme that is effective for at least partial reduction of the infective protein prion in such tissue.

56. A method of degrading an infectious prion protein, comprising (a) heating the infectious prion protein to temperature in a first elevated temperature range that is above 60°C but below the pyrolytic destruction temperature of the prion protein, followed by (b) cooling the infectious prion protein to lower elevated temperature in a second elevated temperature range, and (c) exposing the infectious prion protein to a proteolytic enzyme effective at such lower elevated temperature to degrade the infectious prion protein to a benign degradation product.

57. A method of at least partially degrading infectious prion protein in tissue containing or contaminated with same, by steps including heating the tissue and simultaneously exposing same to a thermal stable proteolytic enzyme, at a temperature that is above 40°C but below the pyrolytic destruction temperature of the prion protein, and for sufficient time, to at least partially degrade the infectious prion protein.

58. A method of processing an animal meat product or by-product to clear BSE-mediating infectious prion protein therefrom, the method comprising treating the animal meat product or by-product with a thermotolerant protease under a temperature that is above 60°C but below the pyrolytic destruction temperature of the prion protein, and for sufficient time, to destroy the BSE-mediating infectious prion protein therein.

59. A tissue composition comprising tissue containing or contaminated with an infectious prion protein and a proteolytic enzyme that is selected from the group consisting of keratinase enzymes, chymotrypsins, pepsins, chymosins, cathepsins, subtilisins, elastases, collagenases,

endopeptidases, peptidases, oligopeptidease, thermolysins, bacillolysin, mycilysins, carboxypeptidases, leucyl aminopeptidases, aminopeptidases, and extremthermophilic proteases.

60. A method of processing of an animal meat product or by-product, comprising treating the animal meat product or by-product with a protease that is effective for destruction of any infectious prion protein associated therewith, at a temperature that is above 40°C but below the pyrolytic destruction temperature of the prion protein, and for sufficient time.

61. A method of treatment for reduction of infective prion protein at a locus contaminated or suspected of being contaminated with infective prion protein, the method comprising the steps of:

(a) heating the locus to a temperature of at least 40°C and for sufficient time to enhance the proteolytic susceptibility of infective prion protein at the locus; and

(b) exposing the heated locus to a proteolytic enzyme that is effective for at least partial reduction of the infective protein prion at such locus.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

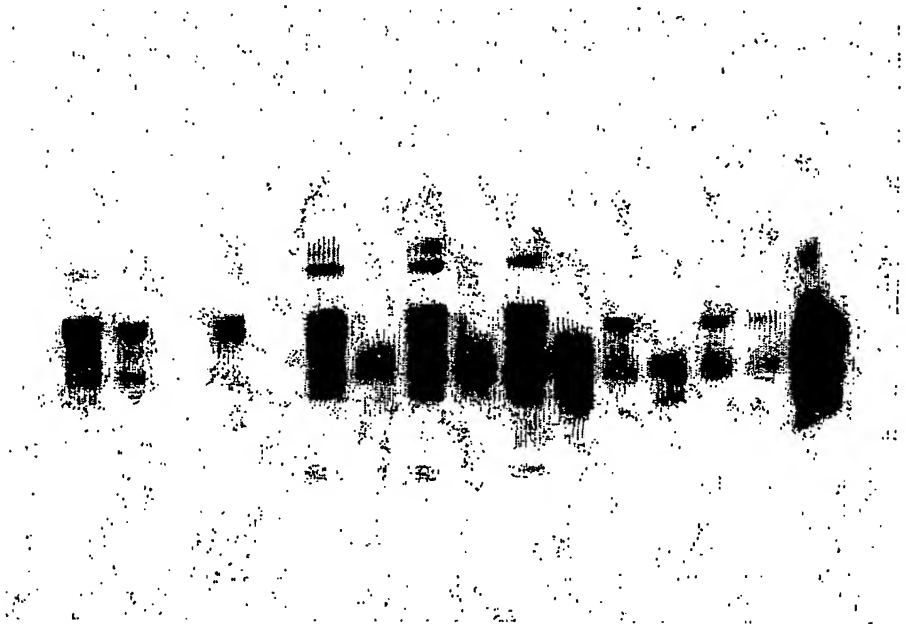

FIGURE 1

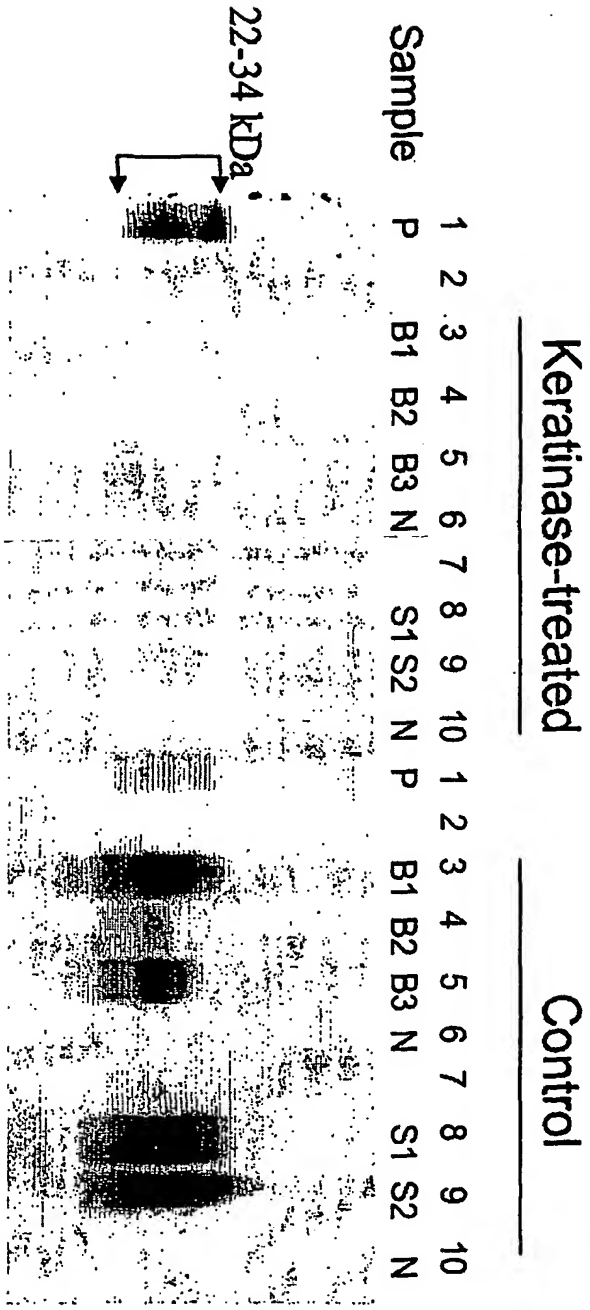
Keratinase-treated
Control
1 2 3 4 5 6 7 8 9 10 1 2 3 4 5 6 7 8 9 10
Sample P B1 B2 B3 N S1 S2 N P B1 B2 B3 N S1 S2 N
22-34 kDa

Figure 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/08982

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : C12Q1/00
US CL : 435/4
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/4, 31,

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y — A	MARSH et al. Physical and chemical properties of the transmissible mink encephalopathy agent. Journal of Virology. 1969, Vol. 3, No. 2, 176-180, especially page 178, column 2, paragraph 2.	1-11,14, 17-37, 39-41, 43-51, 53-61 ——— 12, 13, 15, 16, 38, 42, 52
Y — A	CHO H. J. Inactivation of the scrapie agent by pronase. Canadian Journal of Comparative Medicine, 1983, Vol. 47, pages 494-496, see page 496 column 1.	1, 5, 11, 14, 17, 18, 19, 24, 25, 46, 47 ——— 2-4, 6-10, 12, 13, 15, 16, 20-23, 26-45, 47-61

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
07 February 2003 (07.02.2003)	10 APR 2003
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Ulrike Winkler, Ph.D. Telephone No. 703-308-0196

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PCT/US02/08982

INTERNATIONAL SEARCH REPORT

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y — A	SCHALLER et al. Validation of a western immunoblotting procedure for bovine PrP(Sc) detection and its use as a rapid surveillance method for the diagnosis of bovine spongiform encephalopathy (BSE). Acta Neuropathology. November 1999, Vol.98, No. 5, pages 437-43, especially page 438, column 2, lines 1-3.	1, 5, 11, 14, 17, 18, 19, 24, 25, 46, 47 2-4, 6-10, 12, 13, 15, 16, 20-23, 26-45, 47-61
Y — A	BOLTEN et al. Molecular characteristics of the major scrapie prion protein. Biochemistry. 1984, Vol. 23, pages 2598-5906.	1-11,14, 17-37, 39-41, 43-51, 53-61 12, 13, 15, 16, 38, 42, 52
Y — A	Hunter et al. Attempts to release the scrapie agent from tissue debris. Comparative Pathology. 1967, Vol. 77, pages 301-307, especially Table 5.	39-41, 43-51, 53-61 12, 13, 15, 16, 38, 42, 52
A	McKINLEY et al. A protease-resistant protein is a structural component of the scrapie prion. Cell. November 1983, Vol. 35, pages 57-62,especially page 60, column 2, paragraph 2..	1-61
A	WHO Infection control guidlines for transmissible spongiform encephalopathies. Whorld Health Organization. March 1999, pages 1-38, see especially section 6.	1-61
A, P	Rutala et al. Creutzfeld-Jakob disease: Recomendations for disinfection and sterilization. Clinical Infectious Diseases. 1 May 2001, Vol. 32, pages 1348-1356.	1-61
A	US 5,171,682 (SHIH et al) 15.December 1992 (15.12.1992) see table 4.	1-61

INTERNATIONAL SEARCH REPORT

PCT/US02/08982

Continuation of B. FIELDS SEARCHED Item 3:
WEST, DERWENT, STN, BIOSIS, MEDLINE, EMBASE
prion, enzyme, digestion, degredation, infectivity, sterilization, cleaning, removal.